NOVEL GENOME ANALYZING METHOD

FIELD OF THE INVENTION

This invention relates to a method for determining a
gene expression region for a DNA sequence in which the
nucleotide sequence is already known but its possibility of
being a gene expression region is unclear (specific region)
and a method for determining a gene expression region in an
arbitrary region on a genome or the entire genome by
repeatedly carrying out the above method. The invention
also relates to a genomic gene which was determined to be a
gene expression region by these methods and a protein
encoded by the gene.

While nucleotide sequences of the genome in various
biological species including the human genome composed of
three billion bases are being revealed, development of so-
called post-genome is in progress now. The target of post-
genome is to understand kinds and activities of all
proteins which are produced by a living thing during its
entire life. Also, the main target for human post-genome
is development of novel medicaments based on gene function
analysis (creation of genomic drugs) and establishment of
the basis of tailor-made medical treatments (cf., DeRisi *et
al.*, *Science*, vol. 278, p. 680, 1997).

In the post-genome, particularly the expression mode
of RNA is called transcriptome. In transcriptome analysis,

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identification of all genes on the genome is an important subject. Even if a genomic DNA sequence is revealed, each gene is not identified.

The number of genes on the human genome is estimated to be one hundred thousand, but only six thousand have so far been revealed. Even if some of the remaining genes have important roles, it is difficult to identify them.

(1) For example, in a two-dimensional electrophoresis, rare proteins are easily lost among house-keeping proteins existing in large amounts, so that their discrimination is practically impossible. Also, analysis of cDNA libraries has the same problem; namely, a probability of selecting rare cDNA and subjecting it to nucleotide sequence determination is extremely small. What is more, when identification of all genes is the target, the degree of accomplishment at present cannot be known by these methods.

(2) For example, micro-alley is a technique to identify several thousand kinds of cDNA using tips to which they are linked, but since the linked cDNA molecules are already identified ones, conventionally unknown new genes are not identified.

(3) Also for example, an attempt to newly identify genes using a computer has been reported (cf., Bork *et al.*, *Nature Genet.*, vol. 18, p. 313, 1998), and programs such as GRAIL, HEXON and GENSCAN are provided for carrying out this

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method. However, it goes without saying that

identification of genes based on not assumptions but

experimental data is strongly expected in the transcriptome

analysis.

5   Thus, the object of the invention is to provide a

novel transcriptome analysis method and also to provide a

gene found by such a method and a protein encoded by the

gene.

SUMMARY OF THE INVENTION

10   A first embodiment of the invention made for

achieving the above object is a method for determining

whether or not a continued arbitrary DNA sequence existing

in the genome of an arbitrary biological species, in which

the nucleotide sequence is already known but its

15 possibility of being a gene expression region is unclear

(specific region), is the specific region, which comprises

detecting whether or not a nucleotide sequence that

corresponds to the nucleotide sequence of the region is

present in the RNA of the biological species.

20   A second embodiment of the invention relates to the

first invention, wherein the specific region is a DNA

region of from 100 to 200 bases.

        A third embodiment of the invention relates to the

first or second invention, wherein the detection is

25 comprised of detecting whether or not DNA or RNA is

amplified by the amplification of DNA or RNA based on the

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RNA of the biological species, using an oligonucleotide homologous to a sequence which is comprised of at least 10 or more continued bases and positioned in the 5'-end of the specific region and another oligonucleotide complementary

5   to a sequence which is comprised of at least 10 or more continued bases and positioned in the 3'-end of the specific region.

A fourth embodiment of the invention relates to the third invention, wherein the amplification is an RNA

10   amplification in which, using the oligonucleotides, either one of them having an RNA-transcriptable promoter sequence in its 5'-end, (1) a DNA fragment complementary to a part of RNA of the biological species is synthesized by RNA-dependent DNA polymerase from the either one of the

15   oligonucleotides using the biological species-derived RNA as the template, thereby effecting formation of an RNA-DNA hybrid, (2) a single-stranded DNA fragment is formed by hydrolyzing the biological species-derived RNA of the RNA-DNA hybrid with ribonuclease H, (3) a DNA fragment

20   complementary to the single-stranded DNA fragment is synthesized by DNA-dependent DNA polymerase from the other oligonucleotide using the single-stranded DNA fragment as the template, thereby effecting formation of a double-stranded DNA fragment having a promoter sequence capable of

25   performing transcription of RNA as a part of the RNA of the biological species or RNA complementary to a part thereof,

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(4) an RNA transcription product is formed from the double-stranded DNA using RNA polymerase and then (5) the steps of from (1) to (4) are repeated using the RNA transcription product as the template.

5   A fifth embodiment of the invention relates to the third or fourth invention, wherein the detection of whether or not DNA or RNA is amplified is carried out by a method in which the amplification is carried out in the presence of an oligonucleotide probe which can specifically bind to

10   the DNA or RNA formed by the amplification and is labeled with an intercalating fluorescence dye (provided that the oligonucleotide is a sequence which does not form complementary bonding with any one of the aforementioned oligonucleotides), and changes in a fluorescence

15   characteristic of the reaction solution is measured.

A sixth embodiment of the invention relates to the fifth invention, wherein the probe can perform complementary bonding with at least a part of the sequence of the DNA transcription product or RNA transcription

20   product formed by the amplification, and the fluorescence characteristic changes when compared with the case in which the complex is not formed.

A seventh embodiment of the invention is a method for determining the gene expression region in an arbitrary

25   region on a genome or the entire genome, which comprises

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repeatedly carrying out the method of the first to sixth inventions.

An eighth embodiment of the invention is a genomic gene which was determined to be a gene expression region by the method of the first to seventh inventions. A ninth embodiment of the invention is a protein encoded by the gene of the eighth invention.

BRIEF DESCRIPTION OF THE INVENTION

Fig. 1 shows a relationship between the nucleotide sequence of each of the specific regions 1 to 5 and the complementary bonding position of each of the primers 1F, 1R, 1S, 2F, 2R, 2S, 3F, 3R, 3S, 4F, 4R, 4S, 5F, 5R and 5S.

Fig. 2 shows the non-transcription region and transcription region of the specific regions 1 to 5.

Fig. 3 shows an electrophoresis pattern when 30 cycles of RT-PCR was carried out for 200 ng of mRNA by the method shown in Example 3 using primers for the specific regions 1 to 5.

Figs. 4A, 4B, 4C show respective electrophoresis patterns when 10, 20 and 30 minutes of TRC was carried out for 200 ng of mRNA by the method shown in Example 4 using primers for the specific regions 1 to 5.

Fig. 5 is a graph showing a relationship between the reaction time and the fluorescence intensity ratio which increases with the formation of RNA, when TRC was carried

out for 200 ng of mRNA by the method shown in Example 5 using primers for the specific region 3.

Figs. 6A and 6B show respective electrophoresis patterns when 30 cycles of RT-PCR or 30 minutes of TRC was carried out for 200 ng of mRNA by the method shown in Example 6 using primers for the specific region 3.

Figs. 7A and 7B show respective electrophoresis patterns when 30 cycles of RT-PCR or 30 minutes of TRC was carried out for 0 or 2 ng of mRNA and 0 to 200 ng of genomic DNA, by the method shown in Example 7 using primers for the specific region 3.

Fig. 8 is a graph showing a relationship between the reaction time and the fluorescence intensity ratio which increases with the formation of RNA, when TRC was carried out for 200 ng of genomic DNA by the method shown in Example 7 using primers for a region composed of the specific regions 1, 2 and 3.

DETAILED DESCRIPTION OF THE INVENTION

The following describes the invention in detail.

The method of the invention is applied to a continued arbitrary DNA sequence existing in the genome of an arbitrary biological species, in which the nucleotide sequence is already known but its possibility of being a gene expression region is unclear (specific region). Such a specific region can be set by selecting from published genomic DNA sequences.

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The length of the specific region is not particularly limited but is 200 bases or less, preferably within the range of from 100 to 200 bases. According to the method of the invention, a possibility that the specific region is a

5 gene expression region can be determined only in a case in which the entire portion of an arbitrarily set specific region is included in one exon. Though the number of exons in a gene and the length of each exon greatly vary depending on the kind of gene, one exon containing a

10 termination codon and a poly(A) connecting signal is present in every gene, which is longer than other exons and has more than 400 base pairs. Thus, when an arbitrary genomic region is fragmented into a specific region of 200 base pairs or less, at least one of the fragments is

15 included in the exon and therefore is not overlooked.

According to the present invention, the following detection is carried out by using a continued arbitrary DNA sequence existing in the genome as the specific region, and the gene expression region can be determined on an

20 arbitrary region in the genome or the entire genome by making the arbitrary region or the entire genome into fragments and repeating the detection using each fragment as the specific region.

The invention determines whether or not the specific

25 region is a gene expression region by detecting the presence or absence of a nucleotide sequence which

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corresponds to the nucleotide sequence of the specific region, in RNA of the same biological species. The RNA to be used in the invention is mRNA which is prepared from the same biological species containing the genome to be

5 determined for the gene expression region. Particularly, when the genome is a genome of a higher organism, it is desirable to use various types of mRNA, preferably prepared from all tissues. In that case, the mRNA may be used separately for each tissue or mixed. When the presence of

10 a gene expression region is found in the latter case, subsequent separate use of mRNA of each tissue renders possible finding of a tissue which is expressing the gene in the genome determined to be the gene expression region. The reason that mRNA species can be mixed is as follows.

15 Assuming that average molecular weight of mRNA is 300,000, 1 ng of mRNA will contain 2×10^9 mRNA molecules. Accordingly, even in the case of a gene which is expressed in only one of 1,000 tissues and its expressing quantity is in a ratio of 1/100,000 of mRNA in the tissue, 2×10^4

20 copies are present in 1 μg of the same amount mixture of mRNA respectively obtained from 1,000 tissues including this tissue. As will be described later in Examples, this copy number is sufficiently detectable.

Various method can be applied to the above detection.

25 For example, application of a hybridization method and a nucleic acid amplification method can be exemplified. When

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an amplification method is used, at least two

oligonucleotides (primers) designed based on the specific

region are used in both DNA amplification and RNA

amplification, and one of them is an oligonucleotide

5   homologous to a sequence which is comprised of at least 10

or more continued bases and positioned in the 5'-end of the

specific region and the other is an oligonucleotide

complementary to a sequence which is comprised of at least

10 or more continued bases and positioned in the 3'-end of

10   the specific region. Oligonucleotides of at least 10 or

more bases are used for keeping a specificity regarding

binding of the oligonucleotides to the specific region.

Examples of the nucleic acid amplification method

include a DNA amplification method typified by RT-PCR in

15   which cDNA is synthesized from the mRNA using primers and a

reverse transcriptase and then DNA (DNA comprised of the

specific region) is amplified by a primer elongation

reaction using the primers and a DNA polymerase and the DNA

as the template, and an RNA amplification method in which

20   cDNA complementary to the RNA is synthesized using primers

and a reverse transcriptase and the mRNA as the template,

an elongation reaction of DNA is carried out by binding it

to a promoter primer having a moiety complementary to the

DNA and then RNA (RNA comprised of the specific region) is

25   synthesized in a large amount by allowing an RNA polymerase

to react with the thus synthesized double-stranded DNA.

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The former case is an already broadly and generally known method, and examples of the latter case include NASBA (nucleic acid sequence based amplification) method, 3SR method and the method which will be described later in

5 Examples.

In describing outlines of the NASBA method and the method described in Examples, they are RNA amplification in which, using the oligonucleotides, either one of them having an RNA-transcriptable promoter sequence in its 5'-

10 end, (1) a DNA fragment complementary to a part of RNA of the biological species is synthesized by RNA-dependent DNA polymerase from the either one of the oligonucleotides using the biological species-derived RNA as the template, thereby effecting formation of an RNA-DNA hybrid, (2) a

15 single-stranded DNA fragment is formed by hydrolyzing the biological species-derived RNA of the RNA-DNA hybrid with ribonuclease H, (3) a DNA fragment complementary to the single-stranded DNA fragment is synthesized by DNA-dependent DNA polymerase from the other oligonucleotide

20 using the single-stranded DNA fragment as the template, thereby effecting formation of a double-stranded DNA fragment having a promoter sequence capable of performing transcription of RNA as a part of the RNA of the biological species or RNA complementary to a part thereof, (4) an RNA

25 transcription product is formed from the double-stranded DNA using RNA polymerase and then (5) the steps of from (1)

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to (4) are repeated using the RNA transcription product as the template.

The method described in Examples can be exemplified as particularly desirable detection method from the viewpoints that the determination of the invention can be effected within a short period of time because the amplification is completed within a markedly short time of 10 minutes, that it has a high sensitivity which enables amplification of even several pg of RNA containing the specific region and that the influence of DNA having a possibility of contaminating RNA can be excluded.

The DNA and RNA formed by the above amplification can be detected by an already known detection method such as an electrophoresis, but particularly preferred is a method in which the amplification is carried out in the presence of an oligonucleotide probe which can specifically bind to the DNA or RNA formed by the amplification and is labeled with an intercalating fluorescence dye, and changes in a fluorescence characteristic of the reaction solution is measured. As a matter of course, this probe is a sequence which does not form complementary bonding with the oligonucleotides used in the amplification. Examples of this oligonucleotide probe include those in which an intercalating fluorescence dye is linked to the phosphorus of an oligonucleotide via a linker. In the case of such a suitable probe, when the formed DNA or RNA forms double-

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strand by complementary bonding to a specific region (or a
sequence complementary to the specific region), the
intercalating fluorescence dye intercalates into the
double-stranded moiety and changes its fluorescence

5   characteristic, so that it is not necessary to separate the
probe which did not form complementary bonding (Ishiguro,
T. *et al.*, (1996), *Nucleic Acids Res.*, 24 (24), 4992 -
4997).

Nucleotide sequence of the oligonucleotide probe is
10 not particularly limited with the proviso that it has a
sequence that can perform complementary bonding with the
formed DNA or RNA, but in order to keep a specificity
regarding its bonding to the formed DNA or RNA, it is
desirable that it has about 10 bases which are
15 complementary to at least 10 continued bases existing in
the DNA or RNA. In this connection, when the amplification
is carried out in the presence of the oligonucleotide
probe, it is desirable to modify the hydroxyl group of the
3'-end of the probe chemically (e.g., addition of glycolic
20 acid) in order to suppress elongation reaction in which the
probe is used as a primer.

When the amplification is carried out in the presence
of the oligonucleotide probe as described above, the
detection process of the invention can be carried out in
25 one reaction container at a constant temperature and by one

step, so that its application to automatic operation can be made easily.

Details of the genome analysis method of the invention which is carried out by repeating the gene expression region determination method are as follows, and the method can be applied to any biological species if the genomic sequence is determined. The genome of said biological species is divided, for example, into specific regions each having 200 base pairs. When a nucleic acid amplification is used as the detection method, a primer set containing two oligonucleotides necessary for amplifying each specific region is prepared. In this connection, the number of necessary primers and their sequences vary depending on the nucleic acid amplification method to be employed. Also, it is effective for improving working efficiency to exclude a specific region which is present in a region already known as a gene expression region by previous studies and a specific region which is present in a region that is obviously not a gene expression region based on its DNA sequence, from the objects. Next, the RNA is detected using a primer set for each specific region.

When a genome is analyzed by the method of the invention, all genes of the biological species can be identified. In addition, it becomes possible to determine a protein encoded by a gene of interest, by isolating the gene determined to be a gene expression region and to

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produce the protein making use of the isolated DNA. For example, a nucleotide sequence can be determined by isolating complete length cDNA in the usual way using a nucleic acid amplified by the method of the invention as a

5   probe. By doing this, the genomic structure including the relationship between intron and exon in the gene expression region is revealed. Also, a protein encoded by the gene can be known by isolating cDNA through the screening of a cDNA library in the usual way using the amplified nucleic

10   acid as a probe. In addition, when this protein is expressed, it can be expressed by preparing a recombinant using the cDNA and using a microbial or animal cell as the host in the usual way.

Examples of the invention are given below by way of

15   illustration and not by way of limitation.

EXAMPLE 1

Establishment of regions

In order to show realization possibility of the gene expression region determination method provided by the

20   invention, the following model test was carried out.

As the genomic region, a region composed of 900 base pairs prepared from a G1 strain, a genetically engineered transformed methanol assimilating yeast strain which has been established by the method described by the present

25   inventors in Japanese Patent Application No. 11-188650, was selected. When induced by methanol, the G1 strain

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expresses a human IL-6R-IL-6 fusion protein composed of one polypeptide chain of 397 amino acid residues (cf., Japanese Patent Application No. 11-188650).

As shown in Fig. 1, the region composed of 900 base pairs was divided into five specific regions each having 180 base pairs. Also, mRNA expressing mode of the region already known from Japanese Patent Application No. 11-188650 is shown in Fig. 2. As is evident from Figs. 1 and 2, the specific region 1 (base numbers 1 to 180) contains 159 base pairs of a non-transcription region and 21 base pairs of a transcription region. Each of the specific region 2 (base numbers 181 to 360), specific region 3 (base numbers 361 to 540), specific region 4 (base numbers 541 to 720) and specific region 5 (base numbers 721 to 900) contains only a transcription region.

Oligonucleotide (primer) sets (forward primer; F, reverse primer; R, scissor probe; S) shown in Fig. 1 and SEQ ID NOs;1 to 15 were synthesized for each of the above five specific regions. When DNA amplification (RT-PCR) was carried out, the forward primer and reverse primer among them were used. When RNA amplification (TRC; transcription reverse transcription concerting amplification) was carried out, the forward primer, reverse primer and scissor probe were used. In the TRC, a specific region cannot be amplified when it is not located at the 5'-terminal of mRNA. The scissor probe is an oligonucleotide (DNA) to be

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used in that case for locating the specific region to the 5'-side of mRNA by complementarily binding it to the 5'-side of the specific region and cutting the complementarily bonded region by the action of a ribonuclease.

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EXAMPLE 2

Preparation of mRNA

An mRNA sample of the strain G1 was prepared by the following method.

The strain was inoculated into 3 ml of BMGY (Bacto

10 Yeast Extract 10 g/l, Bacto Peptone 20 g/l, Yeast Nitrogen Base without amino acids 1.34 g/l, 100 mM potassium phosphate buffer, pH 6.0, glycerol 10 g/l and biotin 0.4 mg/l) medium, and cultured at 28°C for 24 hours on a shaker.

15   A 100 µl portion of the culture broth was inoculated into 3 ml of BMGY (Bacto Yeast Extract 15 g/l, Bacto Peptone 30 g/l and other components having the same composition of the above BMGY) medium, and cultured at 28°C for 16 hours.

20   After confirmation of the depletion of methanol, 100 µl of methanol was added to the medium to induce expression of the human IL-6R-IL-6 fusion protein. Two hours after the addition of methanol, the cells were collected and 5 x 10^7 of the cells were immediately frozen with liquid

25 nitrogen.

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They were subjected to cell wall lysis using a
commercially available kit (Yeast cell lysis preparation
kit, mfd. by BIO 01 Inc.) Next, mRNA was prepared using a
commercially available kit (QuickPrep mRNA Purification

5   Kit, mfd. by Amersham Pharmacia).

EXAMPLE 3

Determination of gene expression region by DNA
amplification

Using the mRNA obtained in Example 2, examination was

10 carried out on whether or not the DNA amplification is
specific for a primer derived from a region composed solely
of a gene expression region.

A commercially available kit (RT-PCR beads, mfd. by
Amersham Pharmacia) was used in the RT-PCR.

15 That is, cDNA was synthesized from 200 ng of mRNA by
a 15 minutes of reaction at 42°C using oligo(dT) as a
primer. Next, PCR reaction was carried out using the
forward primer and reverse primer. Using a thermal cycler,
a cycle composed of 95°C for 1 minute, 55°C for 1 minute

20 and 72°C for 2 minutes was repeated 30 cycles spending
about 3 hours. Immediately after the reaction, an
electrophoresis was carried out using 4% agarose which was
then stained with SYBR Green.

As is evident from Fig. 3, amplification was not

25 found by the primer originated from the specific region 1

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but was found by the primers originated from the specific regions 2 to 5.

These results show that the DNA amplification is specific for a primer derived from a region composed solely of a gene expression region, that is, whether or not a continued arbitrary DNA sequence existing in the genome of an arbitrary biological species, in which the nucleotide sequence is already known but its possibility of being a gene expression region is unclear (specific region), is a gene expression region can be determined by detecting the presence or absence of a nucleotide sequence which corresponds to the nucleotide sequence of the region in the RNA of the biological species, by a DNA amplification typified by RT-PCR.

EXAMPLE 4

Determination of gene expression region by RNA amplification

Using the mRNA obtained in Example 2, examination was carried out on whether or not the RNA amplification is specific for a primer derived from a region composed solely of a gene expression region.

(1) Using an RNA dilution solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA), the sample was diluted to 200 ng/5 μl.

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(2) A 20.8 µl portion of a reaction solution of the following composition was dispensed into 0.5 ml capacity tubes and 5 µl of the above RNA sample was added thereto.

Reaction solution composition (each concentration is a concentration in 30 µl of the final reaction solution)

60 mM of Tris-HCl (pH 8.6),

13 mM of $MgCl_2$,

90 mM of KCl,

39 U of RNase inhibitor,

1 mM of DTT,

0.25 mM of each of dATP, dCTP, dGTP and gTTP,

3.6 mM of ITP,

3.0 mM of each of ATP, CTP, GTP and TTP,

0.16 µM of scissor probe,

1 µM of forward primer,

1 µM of reverse primer,

13% of DMSO, and

distilled water for volume adjustment.

(3) This reaction solution was incubated at 65°C for 15 minutes and then at 41°C for 5 minutes, and then 4.2 µl of an enzyme solution having the following composition was added thereto.

Enzyme solution composition (each concentration is a concentration in 30 µl of the final reaction solution)

1.7% of sorbitol,

3 µg of bovine serum albumin,

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142 U of T7 RNA polymerase (mfd. by Gibco),

8 U of AMV reverse transcriptase (mfd. by Takara Shuzo),

distilled water for volume adjusting use.

5   (4) Subsequently, the tubes were kept at 41°C for 10, 20 or 30 minutes. Immediately after the reaction, an electrophoresis was carried out using 4% agarose which was then stained with Cyber Green.

As is evident from Figs. 4A, 4B and 4C, amplification 10 was not found when the primer for the specific region 1 was used in each case of the 10 minute reaction (Fig. 4A), 20 minute reaction (Fig. 4B) and 30 minute reaction (Fig. 4C), but was found when the primers for the specific regions 2 to 5 were used.

15   These results show that the RNA amplification is specific for a primer derived from a region composed solely of a gene expression region, that is, whether or not a continued arbitrary DNA sequence existing in the genome of an arbitrary biological species, in which the nucleotide 20 sequence is already known but its possibility of being a gene expression region is unclear (specific region), is a gene expression region can be determined by detecting the presence or absence of a nucleotide sequence which corresponds to the nucleotide sequence of the region in the 25 RNA of the biological species, by an RNA amplification typified by TRC.

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Also, while the RT-PCR amplification shown in Example 3 required 3 hours even by the use of a thermal cycler, 10 minutes were enough for the amplification by TRC.

EXAMPLE 5

Measurement using oligonucleotide probe labeled with intercalating fluorescence dye

Using the mRNA obtained in Example 2, measurement using an oligonucleotide probe labeled with an intercalating fluorescence dye was carried out.

(1) Using an RNA dilution solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA), the sample was diluted to 200 ng/5 μl.

(2) A 20. 8 μl portion of a reaction solution of the following composition was dispensed into 0.5 ml capacity tubes and 5 μl of the above RNA sample was added thereto.

Reaction solution composition (each concentration is a concentration in 30 μl of the final reaction solution)

60 mM of Tris-HCl (pH 8.6),

13 mM of $MgCl_2$,

90 mM of KCl,

39 U of RNase inhibitor,

1 mM of DTT,

0.25 mM of each of dATP, dCTP, dGTP and gTTP,

3.6 mM of ITP,

3.0 mM of each of ATP, CTP, GTP and TTP,

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0.16 μM of scissor probe (3S, SEQ ID NO;9, the 3'-terminal hydroxyl group is aminated),

1 μM of forward primer (3F, SEQ ID NO;7),

1 μM of reverse primer (3R, SEQ ID NO;8),

25 nM of an oligonucleotide labeled with an intercalating fluorescence dye (YO-3, SEQ ID NO;16, the intercalating fluorescence dye is labeled on the phosphorus between 6th position "T" and 7th position "T" counting from the 5'-terminal, and the 3'-terminal hydroxyl group is modified with glycol group),

13% of DMSO, and

distilled water for volume adjustment.

(3) This reaction solution was incubated at 65°C for 15 minutes and then at 41°C for 5 minutes, and then 4.2 μl of an enzyme solution having the following composition was added thereto.

Enzyme solution composition (each concentration is a concentration in 30 μl of the final reaction solution)

1.7% of sorbitol,

3 μg of bovine serum albumin,

142 U of T7 RNA polymerase (mfd. by Gibco),

8 U of AMV reverse transcriptase (mfd. by Takara Shuzo)

distilled water for volume adjusting use.

(4) Subsequently, the tubes were kept at 41°C and the reaction solution was periodically measured at an

excitation wavelength of 470 nm and a fluorescence

wavelength of 510 nm using a directly measurable

fluorescence spectrophotometer equipped with a temperature

controlling function.

5   Periodical changes in the fluorescence intensity

ratio of the sample (fluorescence intensity value at a

predetermined time / background fluorescence intensity

value) calculated by defining the time of the enzyme

addition as 0 minute are shown in Fig. 5.

10   As shown in Fig. 5, the target RNA contained in 200

ng of mRNA was detected within about 6 minutes. In

addition, the target RNA was detected within about 11

minutes even when the amount of mRNA was reduced to 0.02

ng. Thus, it was shown that quick and high sensitivity

15   measurement can be made by the use of an oligonucleotide

probe labeled with an intercalating fluorescence dye.

EXAMPLE 6

Sensitivity

        Sensitivities of RT-PCR and TRC were compared.

20   Using from 0 to 200 ng of the mRNA obtained in

Example 2, amplification of DNA or RNA was carried out by

30 cycles of RT-PCR by the method shown in Example 3 or by

30 minutes of TRC by the method shown in Example 4.

        As is evident from Figs. 6A and 6B, amplification of

25   0.002 ng of the mRNA was not detected by RT-PCR but was

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detected by TRC.   Thus, it was shown that TRC can achieve

10 times higher sensitivity than RT-PCR.

<div align="center">EXAMPLE 7</div>

Influence of DNA contamination

5   Influences of the contamination of mRNA with DNA in

RT-PCR and TRC were examined.

Firstly, using a commercially available kit (G Nome,

mfd. by BIO 101 Inc.), genomic DNA was prepared from the

cell wall-lysed G1 cell strain obtained by the method

10   described in Example 1. Using from 0 to 200 ng of the DNA

and 0 or 200 ng of the mRNA obtained in Example 2, 30

cycles of RT-PCR was carried out by the method shown in

Example 3, and 30 minutes of TRC by the method shown in

Example 4. As is evident from Figs. 7A and 7B,

15   amplification was observed by RT-PCR when from 2 to 200 ng

of the genomic DNA was present even in the absence of the

mRNA. On the other hand, the amplification did not occur

by TRC in the absence of the mRNA even when from 2 to 200

ng of the genomic DNA was present.

20   Next, relationship between denaturing condition and

amplification of genomic DNA in TRC was examined. Using

200 ng of the genomic DNA, measurement by an

oligonucleotide probe (YO-3, SEQ ID NO;16) labeled with an

intercalating fluorescence dye was carried out by the

25   method shown in Example 5. In this case, 1S (SEQ ID NO;3)

was used instead of 3S (SEQ ID NO;3) as the scissor probe,

<div align="center">25</div>

and 1F (SEQ ID NO;1) was used instead of 3F (SEQ ID NO;7) as the forward primer. The reason for changing the scissor probe and forward primer is to prevent generation of amplification from RNA by changing the amplifying region to a region of 540 base pairs composed of the specific regions 1, 2 and 3 containing the 159 base pair non-transcription region. Also, the constant treating condition of the reaction solution before addition of the enzyme solution (incubation at 65°C for 15 minutes and then at 41°C for 5 minutes) was changed to the following three conditions.

(1) Incubation at 95°C for 15 minutes and then at 41°C for 5 minutes

(2) Incubation at 65°C for 15 minutes and then at 41°C for 5 minutes

(3) Incubation at 41°C for 5 minutes

As is evident from Fig. 8, the time when the fluorescence intensity ratio exceeded 1.2 was about 28 minutes under the condition (1) and about 40 minutes under the condition (2), but its increase was not found under the condition (3).

This result shows that amplification can also occur from DNA by strengthening the denaturing condition. In this case, a change of the treating condition of the reaction solution before addition of the enzyme solution for the condition (3) is convenient in inhibiting amplification from DNA. However, it is expected that the

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amplification from RNA will be inhibited due to formation

of the secondary structure of RNA. In addition, since

periodical changes in the fluorescence intensity ratio are

greatly different between the amplification from RNA and

5   the amplification from DNA, it is markedly easy to make

distinctions between both cases by comparing Fig. 5 with

Fig. 8.

In summing up the above results, it is considered

that a condition of incubating at 65°C for 15 minutes and

10   then at 41°C for 5 minutes is appropriate as the treating

condition of the reaction solution before addition of the

enzyme solution in the mode for carrying out the invention.

Since the use of the method provided by the invention

renders possible revelation of gene expression regions in

15   the entire genome and also of the genomic structure

including the intron-exon relation ship, sequences of all

proteins capable of being expressed in an arbitrary

biological species can be easily determined.

Consequently, according to the present invention, it

20   is considered that understanding of all vital phenomena

becomes possible by making rapid progress in the post-

genome. Also, it is expected that the human post-genome

will lead to the development of novel therapeutic and

diagnostic drugs and also will greatly contribute to the

25   progress of order-made medical treatments. It also will

greatly contribute to the identification of industrially

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useful proteins from microorganisms living under extreme

environmental conditions and to the application thereof.

　　While the invention has been described in detail and

with reference to specific embodiments thereof, it will be

5　　apparent to one skilled in the art that various changes and

modifications can be made therein without departing from

the spirit and scope thereof.

　　This application is based on Japanese patent

applications No. 2000-218737 filed on July 14, 2000, No.

10　2000-263248 filed on August 28, 2000 and No. 2000-334935

filed on October 30, 2000, the entire contents of each of

which are hereby incorporated by reference.